JOURNAL OF VIROLOGY, Sept. 1973, p. 458-463

Vol. 12, No. 3
Printed in U.S.A.

# Studies on the Nucleic Acid Sequences of Kirsten Sarcoma Virus: a Model for Formation of a Mammalian RNA-Containing Sarcoma Virus

EDWARD M. SCOLNICK, ELAINE RANDS, DAVID WILLIAMS, AND WADE P. PARKS

*Viral Lymphoma and Leukemia Branch, Viral Carcinogenesis Branch, National Cancer Institute, Bethesda, Maryland 20014, and Meloy Laboratories, Rockville, Maryland 20850*

Received for publication 26 April 1973

The genetic information contained in the Kirsten and Moloney strains of mammalian RNA-containing sarcoma viruses has been analyzed by RNA·$^3$H-DNA hybridization. Kirsten sarcoma virus has been found to possess two distinct sets of nucleic acid sequences. One set of sequences is contained in murine type C helper virus, and the other set is contained in rat type C helper virus. Moloney sarcoma virus contains sequences of murine type C helper virus but not of rat type C helper virus. The results indicate that Kirsten sarcoma virus arose through a process of recombination between Kirsten murine leukemia virus and nucleic acid sequences found in rat cells. A model is suggested for the formation of transforming type C viruses involving the transduction of oncogenic information.

RNA-containing type C viruses have been classified into two groups: nontransforming viruses which productively infect fibroblast cell cultures called leukemia or helper viruses, and transforming or sarcoma viruses which produce morphological transformation of fibroblasts. Nontransforming helper type C viruses have been frequently isolated from many species (7) and are associated with tumors in the lymphoreticular system, whereas natural isolates of sarcoma viruses are relatively rare. The usual method of isolation of sarcoma viruses in mice and rats has been prolonged animal-to-animal passage of the helper virus and the subsequent isolation from such a stock of two viruses, the original helper and a transforming or sarcoma virus which can produce nonlymphoid soft tissue tumors. Two examples of such experiments were the isolation of Moloney sarcoma virus (Mo-SV) in mice (13) and Kirsten sarcoma virus (Ki-SV) by passage of Kirsten murine erythroblastosis virus (Ki-MuLV) in rats (8).

In many studies (1, 3, 6) on these two transforming virus isolates it has been found that *infectious* sarcoma virus cannot be isolated free of the helper, nontransforming type C virus. However, it has been possible to infect cells with sarcoma virus particles which contain the genetic information necessary for the production and maintenance of cellular transformation but not for complete viral replication (1, 3, 6). These transformed cells do not spontaneously produce infectious transforming virus. When superinfected with helper virus, the cell releases both transforming and nontransforming virus particles.

One of the major questions in cancer biology pertains to the universality of transforming information (7). As an experimental approach to this question, several attempts have been made to characterize viral transforming genetic material (5, 12, 16). One approach to this question is the study of the genetic origin of viral transforming information by nucleic acid hybridization.

The present study compares the RNA nucleic acid sequences present in the Mo-SV- and Ki-SV-transformed nonproducer cells with $^3$H-DNA products made from endogenous reverse transcriptase reactions with murine and rat type C viruses. Evidence of genetically stable transduction of rat type C viral information into Kirsten transforming virus stocks is presented, and a model for the formation of mammalian sarcoma viruses based on transduction is discussed.

## MATERIALS AND METHODS

**Cells.** All cells were grown in Dulbecco's modification of Eagle medium with 10% calf serum (Colorado Serum Co.). The source of all of the cell lines used has been described (4), and the cells used are briefly

described and summarized in Table 1. NRK cells producing endogenous rat type C virus were obtained by iododeoxyuridine induction of these cells (9) and cloning a cell line which continuously produced rat type C virus. Nonproducer lines were derived by limiting dilution of filtered sarcoma virus stocks and isolation of transformed cells in soft agar (3) or Falcon microtest II plates as previously described (14).

**Viruses.** The source and growth of Ki-MuLV and Ki-SV has been described (4). For additional studies to be described below, Ki-MuLV with a known passage history of having been passed only in mice was obtained from Wallace Rowe and Janet Hartley, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Plasma from the original isolation of Ki-SV never grown in tissue culture was obtained from the same source. For the indicated studies, the original Ki-SV was passaged directly into NIH 3T3 mouse cells and NP foci were derived as noted above. Rat helper virus was obtained from NRK cells producing RaLV as noted above.

**Synthesis of virus-specific $^3$H-DNA.** The endogenous reverse transcriptase reaction was used as the source of all products as previously described (4). In addition, all experiments were performed with products purified to insure that the $^3$H-DNA synthesized represented sequences contained in 60–70*S* viral RNA. After 1 to 1.5 h of synthesis, the $^3$H-DNA products were deproteinized with phenol-chloroform, and with ethanol precipitated with 500 $\mu$g of carrier yeast RNA per ml (4). The $^3$H-DNA bound to 70*S* viral RNA was isolated on sucrose gradients as previously described (10, 15). All $^3$H-DNAs were then processed as described (4) to remove RNA and stored at −20 C before use. With a six- to ninefold molar excess of Ki-SV/Ki-MuLV $^3$H-DNA to Ki-SV/Ki-MuLV $^{32}$P-RNA or Ki-MuLV $^3$H-DNA to Ki-MuLV $^{32}$P-RNA, 80 to 90 % of the RNA was resistant to RNase A (16), indicating that the probes contained the majority of sequences present in viral RNA.

**Hybridization.** Hybridization conditions and analysis of the reaction by S1 nuclease have been described (4). The final values of hybridization wth our S1 enzyme were consistently lower (20–30%) than with cesium sulfate analysis; further discussion can be found in earlier work (4). Background values in all cases in the absence of RNA added are 1 to 2% of the input counts per minute. Total cellular RNA was extracted as previously described (4).

TABLE 1. *Cell lines used for hybridization studies*[a]

| Cell line | Description |
|---|---|
| NRK | Normal rat kidney |
| Ki-NRK | Nonproducer rat cell transformed by Ki-SV |
| Ki-NRK + Ki-MuLV | Transformed rat line producing Ki-MuLV and Ki-SV |
| Ki-NIH | Nonproducer NIH mouse cell transformed by Ki-SV |
| Ki-A31 | Nonproducer BALB/c mouse cell transformed by Ki-SV |
| Mo-A31 | Nonproducer BALB/c mouse cell transformed by Mo-SV |
| Mo-NRK | Nonproducer rat cell transformed by Mo-SV |
| NIH 3T3/Ki-MuLV | NIH mouse cell producing Ki-MuLV |
| 3T12 | Transformed BALB/c mouse cell, no exogenous virus added |
| NRK (RaLV) | NRK (Fisher rat) induced with iododeoxyuridine producing endogenous virus |

[a] Virus abbreviation: Ki-MuLV, Kirsten murine leukemia virus; Ki-SV, Kirsten sarcoma virus; Mo-SV, Moloney sarcoma virus; RaLV, rat leukemia virus.

## RESULTS

Virus-specific RNA from Kirsten transformed nonproducer rat cells (Ki-NRK), from rat cells producing rat type C virus (NRK-RaLV), and from rat cells producing Ki-MuLV was measured by hybridization with $^3$H-DNA made from Ki-MuLV grown in NIH 3T3 mouse cells (Fig. 1). Rat cells producing Ki-MuLV contain RNA which readily hybridizes to this product and saturates the reaction at approximately 55% of the input counts per minute. As previously noted (4), the Ki-NRK cell also contains RNA which is detected with this Ki-MuLV $^3$H-DNA, and it saturates at 14% of the input counts per minute, a level lower than the rat cells producing Ki-MuLV. In contrast, the RNA from NRK cells producing an endogenous rat type C virus does not hybridize to the Ki-MuLV probe; in

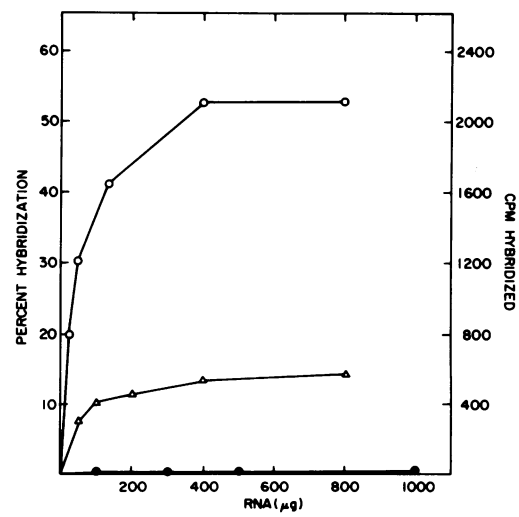


FIG. 1. *Hybridization of various rat cell RNAs with Ki-MuLV $^3$H-DNA. Symbols: ●, NRK producing RaLV; ○, NRK producing Ki-MuLV; △, Ki-NRK.*

studies shown below, this RaLV-producing cell did contain RNA which hybridized to the $^3$H-DNA made from NRK rat virus. The results indicate that the RNA detected in the Ki-NRK cell with Ki-MuLV is not due to rat type C virus that replicates in NRK cells but is associated with the expression of the Kirsten sarcoma genome in these cells. To insure the genetic stability of the nontransforming murine type C sequences associated with the Kirsten sarcoma genome, the sarcoma virus from Ki-NRK was rescued with RaLV transmitted to a new transformed NP cell derived from soft agar. The RNA from this NP cell now also contained the sequences homologous to Ki-MuLV.

The above results suggest that one set of information expressed in Ki-SV NP cells consists of genetically stable sequences contained in murine type C viral RNA. To explore the possibility that other viral or nonviral sequences were expressed in NP cells, the RNA in Kirsten transformed mouse cells (Ki-A31) was hybridized with $^3$H-DNA from Ki-MuLV (same probe as used above) or from Ki-SV/Ki-MuLV grown in rat cells. It is important to note that this preparation of Ki-SV/Ki-MuLV has a three- to fivefold biological excess of transforming virus compared with infectivity titrations of the nontransforming helper virus (4, 16). The hybridization results with these different probes are shown in Fig. 2. The transformed Ki-A31 mouse cells contained RNA which was detectable with either probe. At the point of maximal hybridization, 42% of the input $^3$H-DNA probe hybridized with the Ki-SV/Ki-MuLV probe, and 19% hybridized with the Ki-MuLV probe. The results indicate that Ki-SV DNA probes contain some different sequences than those contained in murine leukemia virus (MuLV) DNA probes. To determine what is represented by these novel sequences in the sarcoma virus probes, additional cells were studied for hybridizable RNA with the Ki-SV/Ki-MuLV probe (Fig. 3). RNA from mouse cells producing Ki-MuLV alone hybridized to only 9 to 10% of the input sarcoma probe even at saturation, whereas the RNA from mouse cells producing Ki-SV and Ki-MuLV saturated at 50 to 53% of the input counts per minute. RNA from either Kirsten nonproducer Ki-A31 or Kirsten nonproducer NIH 3T3 cell saturated at 40 to 42% of the input counts per minute. These results indicate that the $^3$H-DNA from Ki-SV/Ki-MuLV with a biological excess of Ki-SV contains information not contained in mouse cells producing MuLV. Thus Ki-A31 or Ki-NIH cells contain sarcoma *virus*-specific RNA in addition to any RNA of a potential endogenous murine type C virus.

To explore the origin of the additional sequences in Ki-SV, the $^3$H-DNA from the NRK endogenous rat virus was used. This $^3$H-DNA was hybridized to RNA from the same NRK cells as in Fig. 1 producing endogenous rat type C virus, to RNA from 3T12 transformed mouse cells, to NP mouse cells transformed by Moloney sarcoma virus (Mo-A31), to NIH mouse cells producing Ki-MuLV, or to RNA from Ki-A31 or Ki-NIH mouse cells (Fig. 4). The rat cell line NRK, producing its rat type C virus, has RNA which hybridizes to 50 to 55% of the

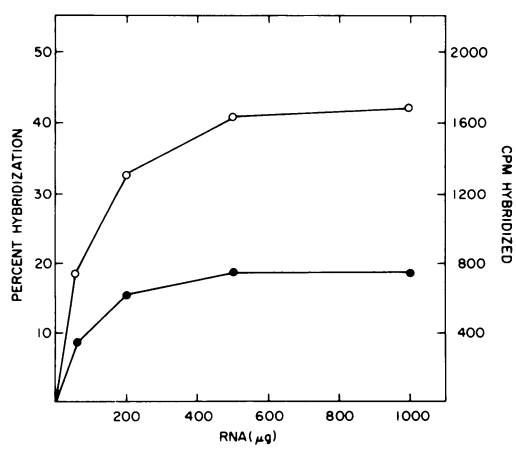


FIG. 2. *Hybridization with Ki-A31 RNA. Symbols: ○, $^3$H-DNA from Ki-SV/Ki-MuLV; ●, $^3$H-DNA from Ki-MuLV.*

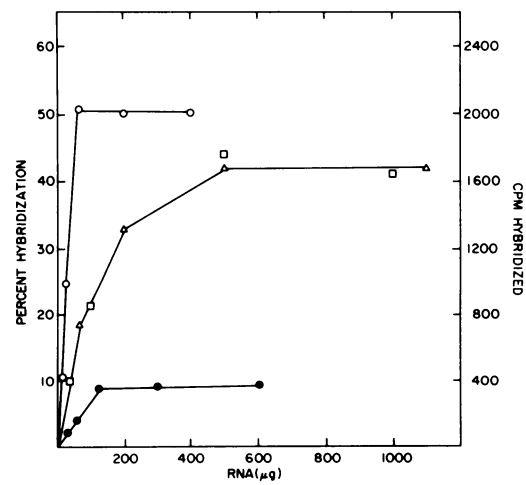


FIG. 3. *Hybridization of Ki-SV/Ki-MuLV $^3$H-DNA with various mouse cell RNAs. Symbols: ○, NIH cells producing Ki-SV/Ki-MuLV; ●, NIH cells producing Ki-MuLV; △, Ki-A31 cells; □, Ki-NIH cells.*

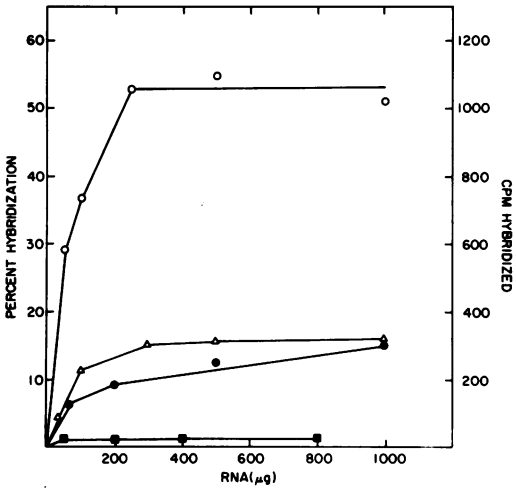


FIG. 4. *Hybridization with RaLV $^3$H-DNA. Symbols: ○, NRK producing RaLV RNA; △, Ki-NIH RNA; ●, Ki-A31 RNA; ■, Mo-A31, or BALB/c 3T12, or NIH producing Ki-MuLV RNA.*

input RaLV $^3$H-DNA counts per minute. In contrast, neither 3T12, Mo-A31, or NIH-Ki-MuLV cells contain RNA which hybridize (<1%) to this same $^3$H-DNA. Both the Ki-NIH and KI-A31 cell do, however, contain RNA which hybridizes to the $^3$H-DNA from rat type C virus, saturating at approximately 15% of the input counts per minute. In other experiments to be reported in detail elsewhere, $C_0t$ analysis using RaLV $^3$H-DNA could also detect rat sequences in Ki-NIH and Ki-A31 cellular DNA but not in Mo-A31 cellular DNA. The results indicate that mouse cells transformed by Ki-SV contain RaLV genetic information, and that this information is not contained in mouse cells transformed by Mo-SV.

To insure the genetic stability of the association of rat sequences with Ki-SV transformed NP mouse cells, the Ki-NIH mouse NP cell was superinfected with Ki-MuLV virus passaged in only mouse cells. The rescued focus-forming virus was transmitted to new NIH 3T3 cells and NP foci isolated from soft agar. These cells again contained comparable levels of RNA and DNA which hybridized to the rat type C $^3$H-DNA product (not shown).

The results with Ki-SV in rat cells indicated that Ki-SV contains murine type C viral nucleic acid sequences. The results in mouse cells indicated that Ki-SV contains additional information also, and that part of this information is contained in RNA found in rat type C helper virus. The studies with Mo-SV-transformed mouse cells also indicated that Mo-SV did not contain rat type C virus information.

Earlier studies had shown that Mo-NRK NP cells did contain information homologous to MuLV (4). The RNA in Mo-NRK cells was re-examined with both Ki-MuLV $^3$H-DNA and Ki-SV/Ki-MuLV $^3$H-DNA (Fig. 5). With the $^3$H-DNA from Ki-MuLV, homologous RNA can be readily detected in Mo-NRK cells but not in NRK cells, or as noted above (Fig. 1), in NRK cells producing rat type C virus. In contrast, with the $^3$H-DNA from Ki-SV/Ki-MuLV which contains the excess of Ki-SV information, Mo-NRK and NRK cells are barely distinguishable, and very little RNA attributable to Moloney sarcoma infection can be detected. In studies not shown, the low level of hybridization observed in NRK with the Ki-SV-containing probe has been found to be due to rat type C RNA in the NRK cells. The results indicate that Mo-SV contains mouse type C nucleic acid sequences and that Ki-SV and Mo-SV differ in their information content.

## DISCUSSION

Transformation of fibroblasts in cell culture by type C sarcoma viruses has provided a unique opportunity to study the mechanism(s) of viral transformation. The involvement of temperature-sensitive viral mutants in the maintenance of transformation (2, 11, 14, 18) suggests the direct involvement of a viral gene in the transformation event. To study further the viral information which encodes for this

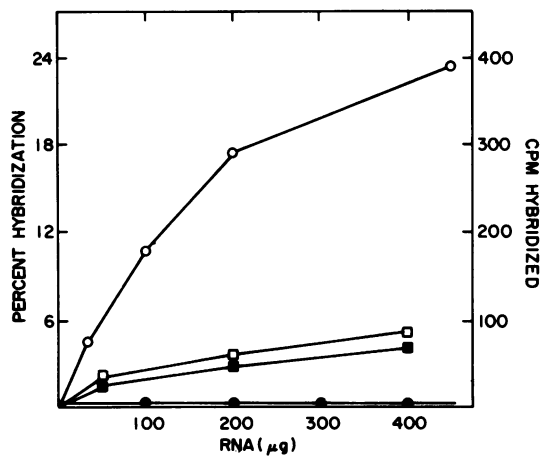


FIG. 5. *Hybridization with NRK versus Mo-NRK RNA. ●, NRK RNA hybridized to Ki-MuLV $^3$H-DNA; ○, Mo-NRK RNA hybridized to Ki-MuLV $^3$H-DNA; □, Mo-NRK RNA hybridized to Ki-SV/Ki-MuLV $^3$H-DNA; ■, NRK RNA hybridized to Ki-SV/Ki-MuLV $^3$H-DNA.*

event it was necessary to be able to distinguish sarcoma virus-specific RNA in sarcoma-transformed nonproducer cells from any potential RNA in these cells contributed by endogenous viral information present in both murine and rat cells. This separation has been possible by the finding that murine and rat type C viral $^3$H-DNA products do not share significant genetic homology (less than 1%). Because of this, we could compare the RNA in mouse and rat nonproducer cells transformed by Ki-SV and distinguish sarcoma virus-specific RNA from that of endogenous murine or rat type C RNA. Ki-SV transformed rat cells, which are not producing virus or known viral proteins, do contain RNA which hybridizes to the $^3$H-DNA product from nontransforming murine virus preparations; the same Ki-MuLV $^3$H-DNA product will not hybridize with rat type C viral RNA. These results indicate that Ki-SV genome contains sequences present in nontransforming murine type C viral RNA. Whether this information is covalently linked to the transforming sequences is not known, nor can we rule out the less likely possibility that these rat cells contain a novel *rat* type C virus which has homology to Ki-MuLV and is induced by infection with Ki-SV.

Studies with the $^3$H-DNA products from Ki-SV/Ki-MuLV compared with Ki-MuLV revealed that Kirsten NP BALB/c cells contain higher levels of novel RNA sequences in addition to RNA related to the nontransforming murine type C viruses. This novel RNA is sarcoma *virus*-specific RNA and not simply that of a potential endogenous virus of murine cells. These results are consistent with the data which showed differences between sarcoma and leukemia viruses by other types of nucleic acid hybridization studies (16). Again we cannot rule out the possibility that BALB/c cells contain a murine virus that has nucleic acid sequences distinct from those detected with Ki-MuLV $^3$H-DNA, is induced by Ki-SV but not Mo-SV, and is homologous to rat virus $^3$H-DNA.

To investigate the origin of these additional sequences, we used a $^3$H-DNA probe from an endogenous rat type C virus. This $^3$H-DNA probe did not hybridize to the RNA from BALB 3T12 cells or BALB/c cells transformed by Mo-SV, or to the RNA from NIH mouse cells producing Ki-MuLV. However, this $^3$H-DNA did hybridize to genetically stable RNA contained in Kirsten NP BALB/c or NIH mouse cells. In addition, this RNA, homologous to rat type C virus, was detected in Kirsten NP mouse cells derived from the virus in the original isolate of Ki-SV. The results indicate that Ki-SV contains two distinct sequences of nucleic acid. One set is homologous to $^3$H-DNA derived from an endogenous reaction of a murine type C virus, and the other is homologous to $^3$H-DNA from an endogenous reaction of rat type C virus. Both of these sequences are a genetically stable part of Ki-SV.

As noted above, the history of isolation of Ki-SV was that Ki-MuLV was passaged in rats, whereas Mo-SV was derived by passage of Moloney leukemia virus in mice. After passage, in each case the nontransforming murine helper virus now also contained a transforming virus, which was later isolated as an NP genome in various cells. With the above data about Ki-SV, the history of isolation from rats suggests a possible model for formation of this transforming virus. During passage of Ki-MuLV in rats, a recombinational event occurred with information contained in rat cells. The process resulted in the formation of a recombinant between Ki-MuLV and sequences in the rat cells. Thus Ki-SV now contains some Ki-MuLV information plus additional information. Part of this additional information clearly has homology with information contained in rat type C viruses. This rat information can be transduced into mouse cells during the process of transmission of Ki-SV from rat cells to mouse cells or mouse cells to mouse cells. Presumably, Mo-SV resulted from similar events in mouse cells; different strains of Mo-SV (3, 6) may then simply reflect varying amounts or kinds of MuLV sequences associated with the transforming information.

It is known from studies on temperature-sensitive mutants of Ki-SV that the constant functioning of a gene product of Ki-SV is needed to maintain transformation (14). The unanswered question is the origin of the information responsible for the maintenance of transformation. One possibility is that this information is due to some unique combination of the sequences detected with the $^3$H-DNA probes made from the nontransforming rat type C virus and mouse type C virus sequences that are detected in Ki-SV transformed cells. Alternatively, other sequences contained in Ki-SV distinct from those in our rat type C and mouse type C $^3$H-DNA products, possibly of rat cellular origin, may have been acquired during formation of Ki-SV which contain the information necessary for the maintenance of transformation.

The current studies provide experimental proof for the hypothesis (17) that transduction of information by type C viruses can occur. In this case the process occurred in the animal and

was accompanied by a transformation event which allowed the transduction event to be recognized. Based on these results, it is reasonable to try to ascertain whether transductional events are also involved in the production of other, naturally occurring sarcomas and leukemias. It is tempting to speculate that type C viruses also have the ability to transduce information in ways that do not lead to oncogenesis, perhaps even more commonly, but there is no assay yet available for these transduction events. Studies with type C viruses are currently in progress to develop assay systems to ascertain if transduction leading to transforming or nontransforming events can be made to occur with regularity in cell culture.

## LITERATURE CITED


1. Aaronson, S. A. and C. Weaver. 1971. Characterization of murine sarcoma virus (Kirsten) transformation of mouse and human cells. J. Gen. Virol. **13**:245-252.
2. Bader, J. P., and N. R. Brown. 1971. Induction of mutations in an RNA tumor virus by an analogue of a DNA precursor. Nature N. Biol. **234**:11-12.
3. Bassin, R. H., N. Tuttle, and P. Fischinger. 1970. Isolation of murine sarcoma virus transformed mouse cells which are negative for leukemia virus from agar suspension culture. Int. J. Cancer **6**:95-107.
4. Benveniste, R., and E. M. Scolnick. 1973. RNA in mammalian sarcoma virus transformed nonproducer cells homologous to murine leukemia virus RNA. Virology **51**:370-382.
5. Duesberg, P. H., and P. K. Vogt. 1970. Difference between the ribonucleic acids of transforming and non-transforming avian tumor viruses. Proc. Nat. Acad. Sci. U.S.A. **67**:1673-1680.
6. Huebner, R. J., J. W. Hartley, W. P. Rowe, W. T. Lane, and W. J. Capps. 1966. Rescue of the defective genome of Moloney sarcoma virus from a noninfectious hamster tumor and the production of pseudotype sarcoma viruses with various murine leukemia viruses. Proc. Nat. Acad. Sci. U.S.A. **56**:1164-1169.
7. Huebner, R. J., and G. Todaro. 1969. Oncogenes of RNA tumor viruses as determinants of cancer. Proc. Nat. Acad. Sci. U.S.A. **64**:1087-1092.
8. Kirsten, W. H., and L. A. Mayer. 1967. Morphologic responses to a murine erythroblastosis virus. J. Nat. Cancer Inst. **39**:311-319.
9. Klement, V., M. O. Nicholson, and R. J. Huebner. 1972. Rescue of focus-forming virus from rat non-productive lines by bromodeoxyuridine. Nature (London) **234**:12-15.
10. Manly, K. F., D. F. Smoler, E. Bromfeld, and D. Baltimore. 1971. Forms of deoxyribonucleic acid produced by virions of the ribonucleic acid tumor viruses. J. Virol. **10**:106-111.
11. Martin, G. S. 1970. Rous sarcoma virus: a function required for the maintenance of the transformed state. Nature (London) **227**:1021-1023.
12. Martin, G. S., and P. H. Duesberg. 1972. The a subunit in the RNA of transforming avian tumor viruses. I. Occurrence in different virus strains. II. Spontaneous loss resulting in non-transforming variants. Virology **47**:494-497.
13. Moloney, J. B. 1966. A virus-induced rhabdomyosarcoma of mice. Nat. Cancer Inst. Monogr. **22**:139-142.
14. Scolnick, E. M., J. Stephenson, and S. A. Aaronson. 1972. Isolation of temperature-sensitive mutants of murine sarcoma virus. J. Virol. **10**:653-657.
15. Schlom, J., and S. Spiegelman. 1971. Simultaneous detection of reverse transcriptase and high molecular weight RNA unique to oncogenic RNA viruses. Science **174**:840-843.
16. Stephenson, J., and S. A. Aaronson. 1971. Murine sarcoma and leukemia viruses: genetic differences determined by RNA-DNA hybridization. Virology **46**:480-484.
17. Temin, H. M. 1971. The protovirus hypothesis: speculation on the significance of RNA-directed DNA synthesis for normal development and for carcinogenesis. J. Nat. Cancer Inst. **46**:3.
18. Toyoshima, K., and P. K. Vogt. 1969. Temperature sensitive mutants of an avian sarcoma virus. Virology **39**:930-931.